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Association of differential β -catenin expression with Oct-4 and Nanog in Oral Squamous Cell Carcinoma and their correlation with Clinicopathological factors and Prognosis

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Abstract

Background: The re-expression of pluripotent markers (Oct-4 and Nanog) and the reactivation of stem cell related pathways in oral carcinoma have been well researched. However, the relationship between the stem cell signaling molecule β -catenin and pluripotent markers Oct-4 and Nanog in oral cancer, is yet to be studied in detail. Therefore, we have investigated the correlation between Oct-4, Nanog and β -catenin in oral squamous cell carcinoma which in turn could provide valuable insight into its prognostic significance.

Methods: The Immunohistochemical analysis was performed for 60 cases of oral cancer to study the expression pattern of Oct-4, Nanog and β -catenin. Whereas, Immunofluorescence analysis was used to investigate the co-localization of β -catenin with Oct-4 and Nanog in oral carcinoma tissues and H314 cell line. Finally, Co-immunoprecipitation analysis was used to study the possible interaction between β -catenin and Oct-4 in oral carcinoma cells.

Results: β -catenin, Oct-4 and Nanog showed significant correlation with lymph node metastasis, stage, grade and prognosis in oral squamous cell carcinoma. Interestingly, a significant positive correlation was found between the expression of Oct-4, Nanog and β -catenin. Moreover, the interaction between β -catenin and Oct-4 was observed in oral cancer.

Conclusions: The positive correlation between Oct-4, Nanog and β -catenin suggests their coordinated role in maintaining proliferation in oral carcinoma cells. The interaction between β -catenin and Oct-4 may be a crucial event in oral carcinogenesis. On the other

hand, β -catenin, Oct-4 and Nanog could be used as independent prognostic markers of oral squamous cell carcinoma.

Keywords: β -catenin; Cancer stem cells; Oct-4; Oral squamous cell carcinoma; Nanog.

Introduction

The transformation from the precancerous to cancerous stage in oral cancer is accompanied with genomic alterations [1]. Apart from the somatic mutation theory of carcinogenesis, the cancer stem cell hypothesis has become the basis for many novel treatment strategies for cancer. The cancer stem cell hypothesis states that a subpopulation of cells in cancerous tissues with tumor inducing capacity as well as stem cell-like characteristics is responsible for carcinogenesis. These cells are termed as cancer stem cells [2]. However, studies on the molecular mechanisms related to cancer stem cells remains obscure. Recently, the expression of stem cell markers Oct-4, Nanog, CD133 and Musashi-1 has been demonstrated in oral squamous cell carcinoma [3, 4]. Additionally, the reactivation of Wnt / β -catenin pathway was observed in oral cancer [5]. Interestingly, several studies show association among β -catenin, Oct-4 and Nanog in embryonic stem cells [6, 7]. Based on this background, we have investigated the expression pattern and relationship between Oct-4, Nanog and β -catenin in oral squamous cell carcinoma, which will provide new insights into the coordinated role of β -catenin, Oct-4 and Nanog in oral cancer. We also analyzed their correlation with clinicopathological factors and prognosis.

In addition to its role in cell-cell adhesion, β -catenin is also a component of the Wnt signaling cascade. Wnt/ β -catenin is one of the major pathways which regulates

proliferation and differentiation of stem cells [8]. In the normal state, β -catenin is mainly present as Axin-APC- β -catenin complex, phosphorylated by GSK3 β . Upon activation, the β -catenin gets separated from the complex, enters into the nucleus and together with Lef/Tcf family of DNA binding proteins, it activates target genes including c-Myc and Cyclin D1. In addition to its role in maintaining stemness of embryonic stem cells, β -catenin was found to have a significant role in several tumors including oral squamous cell carcinoma, particularly the malignant transformation of oral dysplasia is associated with the intracellular expression of β -catenin. [5, 9]. The expression of β -catenin was associated with invasion and metastasis of oral carcinoma cells thereby leading to poor prognosis [5]. Recently, it was found that β -catenin maintains the self-renewal of embryonic stem cells by interacting with Oct-4 and subsequently upregulating Nanog [7]. Since, a relationship between β -catenin and Oct-4 was found in cancer cells and embryonic stem cells, it was evident that β -catenin might maintain the characteristics of cancer stem cells along with Oct-4 [10, 7]. Previously, we have found a positive relationship and interaction between β -catenin and keratinocyte stem/progenitor cell marker Δ Np63 in maintaining proliferation of oral carcinoma cells [5]. Therefore, to extend our knowledge on the relationship between β -catenin and other stem cell markers, we have investigated the correlation between Oct-4, Nanog and β -catenin in oral carcinoma.

Oct-4 is a nuclear protein that belongs to a family of transcription factors containing the POU DNA binding domain [11]. Oct-4 plays a key role in mammalian development and helps in understanding the early events in somatic differentiation of the developing

embryo. It was found that Oct-4 regulates the self-renewal and differentiation in embryonic stem cells [11, 12], while its expression in adult stem cells with an undifferentiated phenotype helps in the maintenance of pluripotency of the population. Oct-4 also helps in identifying adult stem cells which might act as candidates for the initiation of carcinogenesis [13, 14]. Recently, Oct-4 expression was observed in cell lines derived from tissues of the breast, colon, lung and oral cancers. Interestingly, continuous activation of Oct-4 in epithelial cells causes dysplasia which in turn leads to carcinoma [15, 16, 3]. Studies have clearly revealed that the expression of Oct-4 was synchronized with that of Nanog in cancer [17].

Nanog is a homeodomain transcription factor that helps in maintaining the self-renewal capacity in embryonic stem cells. It determines the cell fate of inner cell mass (ICM) during embryonic development. In embryonic stem cells, Nanog is found to act parallel with Oct-4 [18]. Though Nanog was not found in adult tissues, it was observed in several tumors including seminomas, breast cancer, cervical cancer and oral cancer [19, 20, 3]. The co-expression of Nanog with Oct-4 was found in pancreatic carcinogenesis while in lung adenocarcinoma, they were found to induce cancer stem cell-like properties [21, 17]. However, the expression of Nanog was found even in Oct-4 deficient embryo [18]. The overexpression of Nanog promotes recurrence in cisplatin - treated oral squamous cell carcinoma patients and was found to induce the transformation of NIH3T3 cells [22, 23]. Moreover, Nanog was found to regulate the self-renewal potential in hepatocellular cancer stem cells through the Insulin-like growth factor (IGF) pathway [24]. As mentioned earlier, β -catenin upregulates Nanog expression in embryonic stem cells by

binding with the Lef/Tcf element in the enhancer region of the human NANOG gene [25].

In order to support the role of β -catenin in maintaining proliferation of oral cancer cells, the expression pattern of the keratinocyte differentiation marker Involucrin was also investigated in oral cancer. Involucrin is a cell envelope protein identified as a marker for terminal differentiation of keratinocytes. Involucrin expression in poorly differentiated tumors is much less than in cells of well differentiated tumors. Involucrin expression is also dependent on the level of hypoxia in the tumor tissues [26]. During the *in vitro* differentiation of human embryonic stem cells to keratinocytes, the expression of Involucrin was found only during the initiation of keratinocyte differentiation [27].

Eventhough several studies show the involvement of stem cell markers and signaling molecules in oral squamous cell carcinoma, the association between Oct-4, Nanog and β -catenin has not yet been studied. Additionally, the correlation between Oct-4, Nanog and clinicopathological factors has not been extensively studied in oral cancer. Hence, studying the expression pattern of these proteins will uncover novel facets of their role in oral squamous cell carcinoma. Therefore, we have investigated the expression pattern and association between Oct-4, Nanog and β -catenin in oral carcinoma. We have also analyzed the prognostic significance of these proteins in oral cancer which will in turn play a pivotal role while determining therapeutic strategies.

Materials & Methods:

Tissues & Cell line

The samples used in this study were obtained from 60 patients, aged between 45 and 70, with a mean age of 59.3. The tissue samples were obtained with proper consent from the patients referred to the Department of Surgical Oncology, Government Royapettah Hospital, Chennai. Six normal samples were obtained from the patients undergoing orthodontic surgery. The range of follow-up was 14 to 48 months with a mean follow-up period of 31.9. Histopathological parameters were assessed according to the criteria illustrated by Pindborg et al [28]. 10% buffered formalin was used for fixing the tissues and they were processed for paraffin embedding. 4 μ m sections were mounted on coated glass slides. Clearance for the study was obtained from the Hospital Medical Board with permission from the Directorate of Medical Education, Government of Tamil Nadu, India.

The cell-bearing slides of H314 cell line (derived from a poorly differentiated tumour of the floor of the mouth) [29] and cell-bearing coverslips of CAL27 cell line (Human tongue squamous cell carcinoma) [30] were obtained as gift from Dr. Angela Hague and Dr. Karl Kingsley respectively.

Immunohistochemistry

Sections were deparaffinized in xylene, rehydrated through graded alcohols and the antigen retrieval was done by heating the slides in 0.1M sodium citrate buffer (pH 6.0) in a microwave oven. After cooling, the endogenous peroxidase activity was blocked by placing the slides in 3 % hydrogen peroxide for 10 minutes. The non-specific binding sites were blocked with 0.3% Bovine Serum Albumin (BSA) for 30 minutes at room

temperature. The sections were then incubated (overnight at 4°C) with mouse monoclonal anti-Oct-4 (Santa Cruz Biotech, USA; sc-5279), rabbit polyclonal anti- β -catenin (Santa Cruz Biotech., USA; sc-7199) and goat polyclonal anti-Nanog (Santa Cruz Biotech., USA; sc-30331) in a dilution of 1:200 for all the above mentioned antibodies. Additionally, the cases showing intracellular expression of β -catenin were also incubated with mouse monoclonal anti-Involucrin antibody (a kind gift from Dr.Fiona Watt, UK; SY5; 1:100). The slides were immunostained using the respective horseradish peroxidase conjugated secondary antibodies (Invitrogen, USA). The chromogen 3, 3'-diaminobenzidine was then used as a substrate for localizing the antibody binding. After Counter-staining with haematoxylin, the sections were mounted and viewed under microscope. Negative controls without primary antibody were included for each staining. The deparaffinization step was omitted in the case of CAL27 cells. The immunostained sections were scored according to the percentage of stained cells in more than three random areas of cancer tissue as examined in 200x magnification. Two independent observers without prior knowledge on the patient's clinicopathological data assessed the immunostained slides. The staining was assessed as 0 (negative), no staining or staining in less than 5% of cells; 1⁺ (mild), staining in 5-15% of carcinoma cells; 2⁺ (moderate), staining in 16-25% of carcinoma cells; 3⁺ (intense), staining in >25% of carcinoma cells. The staining was also graded as Negative (includes categories 0 and 1⁺) and Positive (includes categories 2⁺ and 3⁺). Additionally, the localization of β -catenin was considered as 0, negative; M, membranous and I, intracellular, to study the correlation of β -catenin localization with the expression of Oct-4 and Nanog. In case of disagreement, the slides were re-evaluated by the two observers and a consensus was reached after discussion.

Immunofluorescence

The Immunofluorescence analysis was done in carcinoma tissues and in H314 cell line.

The sections were deparaffinized in xylene, rehydrated and fixed for 5 minutes. The cells were then washed with Phosphate Buffer Saline (PBS) and permeabilized with 0.1% Triton X-100. The cells were then blocked with 1% BSA for 30 minutes and incubated (1 hour) with a combination of anti- β -catenin / anti-Oct 4 and anti- β -catenin / anti-Nanog antibodies. In addition, the H314 cells were also incubated with a combination of anti- β -catenin / anti-Involucrin antibodies. After washing with PBS, the slides were incubated for 2 hours with respective FITC conjugated secondary antibodies for Oct-4, Nanog and Involucrin and Alexa Fluor 594 (red) conjugated secondary antibody for β -catenin. After incubation, the slides were washed with PBS, mounted and examined under confocal fluorescence microscope (Leica TCS SP2, Leica Microsystems, Germany). Fluoroshield Mounting medium with DAPI (Sigma, USA) was used for DAPI staining and the staining was done according to the manufacturer's protocol.

Western blot and Co-immunoprecipitation

Western blot and co-immunoprecipitation analysis were done in 16 cases including normal (6) and carcinoma (10). The protein lysates were prepared as described by Ratovitski et al [31] and immunoblotted as input control. The protein concentrations were measured by Lowry's method [32]. Samples were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and the membranes were blocked in Tris-buffered saline containing 5% skim milk and 0.05% Tween-20. The membranes were then incubated with anti- β -catenin, anti-Oct-4 and anti-Nanog antibodies and the western blot was performed using the respective horseradish

peroxidase (HRP) conjugated secondary antibodies. The signals were detected using Amersham ECL plus kit according to the manufacturer's protocol. For co-immunoprecipitation, the β -catenin immune complex was prepared as described in our previous report [5] and the immune complex was then incubated with 20 μ l of protein-A-sepharose beads (Sigma) for 30 minutes. The immunocomplex were centrifuged, washed in homogenizing buffer and the pellets were resuspended in sample buffer. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was then incubated with anti-Oct 4 antibody and the Immunoblot analysis was performed as described above. The cell lysates were immunoprecipitated with IgG (Santa Cruz Biotech, USA) and used as negative control.

Statistical analysis

The relationship between clinicopathological parameters and the expression of proteins were analyzed using Chi square test. The correlation between β -catenin, Oct-4 and Nanog was statistically evaluated using Spearman's correlation analysis. The overall and disease-free survival curves were constructed by the Kaplan-Meier method and the log-rank test was used to assess the difference between resulting curves. The duration of disease-free survival was between the date of treatment to the date of recurrence or metastasis or disease related death. The cases were censored either at the date of death for patient who died during the trial or at the date of last examination for patient who lost the follow-up. The Univariate and Multivariate survival analyses were performed using Cox proportional hazards regression model. The statistical analyses were performed using Acastat Statistical software, version 6.1[Acastat Software, USA] and the survival analysis was performed using SPSS software (SPSS for windows 14.0, SPSS Inc., Chicago,

Illinois). The $p < 0.05$ was considered significant for all the statistical analyses. Due to the exploratory nature of the study, we did not adjust for multiple tests.

Results

Expression pattern of β -catenin, Oct-4 and Nanog in oral squamous cell carcinoma tissues

β -catenin

The expression pattern of β -catenin, Oct-4 and Nanog are presented in Table 1. Out of 60 cases of oral carcinoma, 10 cases showed negative and 9 cases showed mild staining for β -catenin. In addition, 11 and 30 cases showed moderate and intense staining respectively. Aberrant localization of β -catenin was observed in oral carcinoma cells (Figure 1A (a)). Among the 60 cases of oral squamous cell carcinoma, 30 cases showed intracellular expression of β -catenin. The expression of β -catenin did not associate with age, gender and location of tumors ($P > 0.05$). However, significant correlation was found between stage ($\chi^2 = 6.163$; $P < 0.01^*$), lymph node metastasis ($\chi^2 = 8.155$; $P < 0.004^{**}$), histological grade ($\chi^2 = 6.181$; $P < 0.04^*$) and β -catenin expression (Table 2). Also, intense expression of β -catenin was found in CAL27 cells (Figure 2A (a)).

Oct-4

Differential expression pattern of Oct-4 was observed in oral carcinoma tissues. The nuclear expression of Oct-4 was considered as 'positive staining'. The expression of Oct-4 was predominantly observed in the nucleus but was also found in the cytoplasm of some carcinoma cells (Figure 1A (b)). Intense staining of Oct-4 was observed in 18 cases of oral carcinoma, whereas, 20 cases showed negative staining for Oct-4. Among the remaining 22 cases, 12 and 10 cases showed mild and moderate staining respectively

(Table 1). The expression of Oct-4 was significantly correlated with stage ($\chi^2 = 5.725$; $P < 0.01^*$). Particularly, the Oct-4 expression was higher in stage III-IV when compared with stage I-II. Additionally, Oct-4 staining was significantly higher in lymph node metastasis positive cases than that in lymph node metastasis negative cases ($\chi^2 = 6.907$; $P < 0.009^{**}$). Oct-4 expression also showed a significant difference between well, moderate and poorly differentiated tumors ($\chi^2 = 15.265$; $P < 0.001^{**}$). There was no significant difference between age, gender, location of tumor and Oct-4 expression (Table 2). The CAL27 cells showed increased expression of Oct-4 (Figure 2A (b)).

Nanog

The expression of Nanog which was primarily localized in the nucleus also showed cytoplasmic staining in some carcinoma cells (Figure 1B (d)). However, similar to Oct-4, nuclear expression of Nanog was considered as 'positive staining'. Out of 60 cases of oral carcinoma, 17 and 14 cases showed negative and mild staining for Nanog respectively. The expression of Nanog was moderate in 10 cases and intense in 19 cases of oral squamous cell carcinoma (Table 1). The expression of Nanog was significantly higher in stage III-IV when compared with stage I-II ($\chi^2 = 6.877$; $P < 0.009^{**}$). Statistically significant association was not found between age, gender, location of tumor and Nanog expression. Nanog expression was significantly correlated with lymph node metastasis ($\chi^2 = 8.210$; $p < 0.004^{**}$). Moreover, expression of Nanog was associated with histological grade of oral carcinoma ($\chi^2 = 18.204$; $P < 0.001^{**}$) (Table 2). Furthermore, intense expression of Nanog was observed in CAL27 cells (Figure 2A (c)).

Association of β -catenin expression with Oct-4, Nanog and Involucrin in oral squamous cell carcinoma

The increased expression of β -catenin was found to be associated with the expression of Oct-4 in oral squamous cell carcinoma (Figure 1A (a) and (b)). In addition, Spearman's correlation analysis revealed a significant positive association between the expression pattern of β -catenin and Oct-4 ($P < 0.001^{**}$, $r_s = 0.80$) in oral carcinoma. Interestingly, cases with high β -catenin expression showed increased expression of Nanog. In addition, statistically significant positive correlation was found between the expression of β -catenin and Nanog ($P < 0.001^{**}$, $r_s = 0.71$) in oral squamous cell carcinoma (Figure 1B (c) and (d); Table 3).

Oct-4 expression was significantly higher in cases showing intracellular expression of β -catenin. Also, a positive linear relationship was found between localization of β -catenin and Oct-4 expression ($P < 0.001^{**}$, $r_s = 0.71$). Moreover, Nanog expression was also higher in cases showing intracellular expression of β -catenin with a significant positive correlation ($P < 0.001^{**}$, $r_s = 0.67$) obtained using Spearman's correlation analysis (Table 4). Furthermore, Involucrin expression was not found in carcinoma tissues showing intracellular expression of β -catenin (Figure 1C (e) and (f)).

Co-localization of β -catenin with Oct-4, Nanog and Involucrin in oral squamous cell carcinoma.

Double-immunofluorescence analysis was done in oral carcinoma tissues and H314 cell line. The expression of Oct-4 was predominantly found in oral carcinoma and H314 cells showing intracellular expression of β -catenin supporting the results of the correlation analysis (Figure 2A). In addition, the expression of Nanog was higher in oral carcinoma and H314 cells in which intracellular localization of β -catenin was observed (Figure 2A).

However, negative expression of Involucrin was observed in H314 cells showing intracellular expression of β -catenin (Figure 2B).

Immunoblot and Co-immunoprecipitation analysis

Western blot analysis was done to confirm the results obtained in Immunohistochemical and Immunofluorescence analyses. Western blot analysis revealed increased band intensity for β -catenin in carcinoma samples when compared with normal samples (Figure 2C (a)). The expression of both Oct-4 and Nanog were detected only in carcinoma samples and not in normal samples (Figure 2C (b) and (c)). On the other hand, the interaction between β -catenin and Oct-4 was studied using co-immunoprecipitation analysis (Figure 2C (d)). The interaction between β -catenin and Oct-4 was not observed in normal cases. Whereas, out of 10 carcinoma cases, 8 cases showed interaction between β -catenin and Oct-4 (Supplement table 1).

Survival analysis

The survival curves constructed using the Kaplan-Meier method and compared by log-rank test showed that the β -catenin, Oct-4 and Nanog positive patients had poorer survival rates than those patients with negative expression in overall and disease-free survival (Figure 3 and 4). Additionally, the survival of patients with intracellular expression of β -catenin was worse when compared with other patients in overall and disease-free survival (Figure 3d and 4d). Interestingly, β -catenin and Oct-4 double positive cases had a significant shorter survival time than other cases (Figure 3e and 4e). Moreover, β -catenin and Nanog double positive cases were significantly correlated with worse outcome in overall and disease-free survival (Figure 3f and 4f). Extent of lymph

node metastasis, clinical stage and poor differentiation status were significantly correlated with worst survival (Table 5; Supplement figure 1).

The Univariate Cox Proportional Hazards Regression model revealed that high clinical stage, lymph node metastasis, high β -catenin expression, high Oct-4 expression and high Nanog expression had prognostic significance in overall and disease-free survival of patients with oral squamous cell carcinoma (Table 6). In addition, the Multivariate Cox Proportional Hazards Regression model indicate that clinical stage, β -catenin, Oct-4 and Nanog were independent prognostic factors in overall and disease-free survival of oral carcinoma patients (Table 7).

Discussion

Evidences to substantiate the cancer stem cell hypothesis have been emerging rapidly in various tumors. Several studies highlight the significant role of pluripotent markers and stem cell pathways in carcinogenesis. However, the molecular basis for the maintenance of cancer stem cell properties has not been extensively studied in oral squamous cell carcinoma. In this study, we have investigated the expression pattern and correlation between Oct-4, Nanog and β -catenin in oral squamous cell carcinoma and CAL27 cell line. We also analyzed the association between the expression of these proteins with clinicopathological factors and prognosis in oral cancer. On the other hand, we have studied the co-localization of β -catenin with Oct-4 and β -catenin with Nanog in oral squamous cell carcinoma tissues and H314 cell line.

β -catenin signaling was found to play a significant role in oral squamous cell carcinoma. The absence of membranous and subsequent intracellular expression of β -catenin was

found to be a key event in oral cancer [9]. In the present study, absence of membranous and intense intracellular expression of β -catenin was mainly observed in oral carcinoma and CAL27 cells which were concurrent with earlier reports by Ishida et al [9] and Kobayashi et al [33]. This suggests that the β -catenin pathway might be re-activated and involved in oral carcinogenesis. Consistent with our previous findings [5], the expression of β -catenin was significantly correlated with lymph node metastasis, stage and grade of oral squamous cell carcinoma. This indicates that the expression of β -catenin may be related to the invasion and differentiation status in oral carcinoma cells. In addition, the expression of β -catenin seemed independent of age, gender and location of the tumor since the statistical significance was not reached.

Oct-4, which plays a crucial role in maintaining the pluripotent state of embryonic stem cells, is not expressed in normal mature cells [34, 14]. Identification of Oct-4 in tumour tissues and cell lines is indicative of the reactivation of stem cell related pathways in cancer [14]. Recently, Oct-4 expression was also found in oral cancer stem-like cells [3]. Consistent with the findings of Ge et al [35] and Chiou et al [3], nuclear expression of Oct-4 was mainly observed in oral carcinoma and CAL27 cells. However, cytoplasmic expression of Oct-4 was also observed in some carcinoma cells, similar to the expression pattern observed in rectal adenocarcinoma [36] and seminomas [37]. These results suggest the need for investigating the localization of Oct-4 in oral cancer. As mentioned by Ge et al [35], the expression of Oct-4 was significantly correlated with lymph node metastasis. This suggests that Oct-4 may be related to invasion and progression in oral cancer. Moreover, in line with the findings of Chiou et al [3], significant association was found between Oct-4 expression and the stage and grade of oral carcinoma. This indicates

that the expression of Oct-4 may be related to the differentiation status of oral carcinoma cells.

Similar to Oct-4, Nanog is a pluripotent marker essential to maintain the self renewal of embryonic stem cells. Recently, the expression of Nanog was found in several tumors including oral squamous cell carcinoma [18-20, 3]. Interestingly, together with Oct-4, Nanog was found to induce cancer stem cell properties in lung adenocarcinoma [17]. In the present study, expression of Nanog was predominantly restricted to the nucleus in oral carcinoma and CAL27 cells. However, cytoplasmic staining was also found in some oral carcinoma cells as in cervical cancer [20]. These results suggest the need for investigating the specific function of Nanog based on its localization in oral cancer cells.

Consistent with the findings of Chiou et al [3], the current study revealed that the expression of Nanog was higher in poorly differentiated tumours and in higher stages of oral carcinoma. This indicates that the expression of Nanog may be related to aggressiveness and differentiation of oral carcinoma cells. In addition, the expression of Nanog was significantly correlated with lymph node metastasis which was in agreement with the findings of Meng et al [38]. This suggests that the expression of Nanog may be related to the metastatic potential of oral carcinoma cells.

β -catenin was found to maintain the self renewal potential of embryonic stem cells along with Oct-4 and Nanog [7]. In this study, we have found a significant association between Oct-4, Nanog and β -catenin in oral carcinoma. In addition, the intracellular expression of β -catenin was positively correlated with Oct-4 and Nanog expression in oral cancer. Co-expression of β -catenin with Oct-4 and β -catenin with Nanog was also observed in oral carcinoma tissues and H314 cells. These results suggest a significant relationship among

β -catenin, Oct-4 and Nanog in maintaining proliferation and self renewal of oral carcinoma cells. Consistent with studies by Kamino et al [39] and Chou et al [26], we have found a decreased expression of Involucrin in oral squamous cell carcinoma tissues. Particularly, negative expression of Involucrin was observed in oral carcinoma cells which showed intracellular expression of β -catenin. This suggests that β -catenin signaling may help in maintaining proliferation of oral carcinoma cells by downregulating differentiation. As in embryonic stem cells [6, 7], the possible interaction between β -catenin and Oct-4 was observed in oral squamous cell carcinoma tissues. This result explores the coordinated function of β -catenin and Oct-4 in oral carcinoma cells. However, further studies will help to determine the accurate role of their correlated expression which can further be applied for therapeutic target selection.

In this study, the expression of β -catenin (particularly intracellular), Oct-4 and Nanog showed significant correlation with poor survival in oral squamous cell carcinoma as mentioned by Odajima et al [40] for β -catenin and Chiou et al [3] for Oct-4 and Nanog. This suggests that the reactivation and dysregulated expression of these proteins may be related to progression and aggressiveness in oral squamous cell carcinoma. Interestingly, β -catenin/Oct-4 and β -catenin/Nanog double positive cases showed worst survival in oral cancer. This indicates that the co-expression of β -catenin with Oct-4 and β -catenin with Nanog might be a valuable indicator of high risk cases. Moreover, the intracellular expression of β -catenin significantly correlates with poor survival in oral carcinoma which explores that the activated β -catenin signaling may contribute to cancer related mortality in oral carcinoma patients. By univariate and multivariate analysis, we have found that β -catenin, Oct-4 and Nanog have prognostic value in oral squamous cell

carcinoma. Also, the present study showed that β -catenin, Oct-4 and Nanog might be used as independent prognostic markers of oral cancer which is concurrent with the findings of Odajima et al [40] for β -catenin and Chiou et al [3] for Oct-4 and Nanog.

In conclusion, the expression pattern of β -catenin, Oct-4 and Nanog in oral carcinoma and CAL27 cells reveal the involvement of these proteins in oral carcinogenesis. The positive correlation and co-expression of β -catenin with Oct-4 and β -catenin with Nanog suggests their coordinated role in oral squamous cell carcinoma. Also, it indicates that the β -catenin signaling might act synergistically with Oct-4 and Nanog in regulating proliferation and differentiation of oral carcinoma cells. Interestingly, the interaction between β -catenin and Oct-4 in oral carcinoma cells might be a crucial event in oral carcinogenesis which requires further investigation. Additionally, the prognostic value of β -catenin, Oct-4 and Nanog in oral squamous cell carcinoma, particularly, the co-expression of β -catenin with Oct-4 and β -catenin with Nanog may be used to identify high-risk cases. Further studies on larger sample size and on the tumor inducing potential of β -catenin, Oct-4 and Nanog positive oral carcinoma cells could provide valuable information on the role of these proteins in oral carcinogenesis, their value as prognostic markers and their therapeutic significance.

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Conflict of interest: We have no conflict of interest.

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Figure legends

Figure 1: Immunostaining of β -catenin, Oct-4 and Nanog in oral squamous cell carcinoma. A) Serial section of oral carcinoma tissue showing positive staining for β -catenin (a) (Inset: carcinoma cells showing membranous expression of β -catenin) and Oct-4 (b). B) Serial section of oral carcinoma tissue showing positive staining for β -catenin (c) (Inset: carcinoma cells showing nuclear expression of β -catenin) and Nanog

(d). C) Serial section of oral carcinoma tissue showing positive staining for β -catenin (e) and Negative staining for Involucrin (f). Bar = 50 μ m.

Figure 2: Co-localization of β -catenin, Oct-4 and Nanog was analyzed in CAL27 cells and carcinoma tissues using Immunofluorescence analysis. (A) Immunodetection of β -catenin (a), Oct-4 (b) and Nanog (c) in CAL27 cell line. Section of oral carcinoma tissue showing positive staining for Oct-4 (d), intracellular expression of β -catenin (e) and expression of Oct-4 in cells showing intracellular expression of β -catenin (f); Arrows indicate co-localization of β -catenin and Oct-4 in oral carcinoma cell. Section of oral carcinoma tissue showing positive staining for Nanog (g), intracellular expression of β -catenin (h) and expression of Nanog in cells showing intracellular expression of β -catenin (i); Arrows indicate co-localization of β -catenin and Nanog in oral carcinoma cell.

H314 cells showing positive staining for Oct-4 (j), β -catenin (k) and expression of Oct-4 in β -catenin positive H314 cells (l); Arrows indicate co-localization of β -catenin and Oct-4 in H314 cell.

H314 cells showing positive staining for Nanog (m), β -catenin (n) and expression of Nanog in β -catenin positive H314 cells (o); Arrows indicate co-localization of β -catenin and Nanog in H314 cell. Bar = 50 μ m.

(B) H314 cells showing positive staining for β -catenin (a) and negative staining for Involucrin (b). Bar = 50 μ m.

(C) a. Western blot for β -catenin (~ 92 kDa). Input controls for β -catenin in normal (Lane 1) and carcinoma (Lane 2) tissues; IgG controls for β -catenin in normal (Lane 3) and carcinoma (Lane 4) tissues.

- b. Western blot for Oct-4 (~ 45 kDa). Input controls for Oct-4 in normal (Lane 1) and carcinoma (Lane 2) tissues; IgG controls for Oct-4 in normal (Lane 3) and carcinoma (Lane 4) tissues.
- c. Western blot for Nanog (~ 40 kDa). Input controls for Nanog in normal (Lane 1) and carcinoma (Lane 2) tissues; IgG controls for Nanog in normal (Lane 3) and carcinoma (Lane 4) tissues.
- d. Protein lysates were immunoprecipitated with anti- β -catenin and immunoblotted with anti-Oct-4.
- e. Western blot for β -actin (control).

Figure 3: Overall survival of patients with oral squamous cell carcinoma according to the expression of β -catenin ($p = 0.0036$) (a), Oct-4 ($p = 0.0001$) (b), Nanog ($p = 0.0001$) (c), β -catenin localization ($p = 0.0001$) (d), β -catenin + Oct-4 (double positive; $p = 0.0001$) (e) and β -catenin + Nanog (double positive; $p = 0.0001$) (f) calculated by Kaplan-Meier method. Negative – includes categories 0 and 1^+ ; Positive – includes categories 2^+ and 3^+ ; Intra. – intracellular; B+ / O+ - β -catenin and Oct-4 double positive; B+ / N+ - β -catenin and Nanog double positive.

Figure 4: Disease-free survival of patients with oral squamous cell carcinoma according to the expression of β -catenin ($p = 0.0395$) (a), Oct-4 ($p = 0.0001$) (b), Nanog ($p = 0.0012$) (c), β -catenin localization ($p = 0.0001$) (d), β -catenin + Oct-4 (double positive; $p = 0.0001$) (e) and β -catenin + Nanog (double positive; $p = 0.0001$) (f) calculated by Kaplan-Meier method. Negative – includes categories 0 and 1^+ ; Positive – includes

categories 2⁺ and 3⁺; Intra. – intracellular; B+ / O+ - β -catenin and Oct-4 double positive; B+ / N+ - β -catenin and Nanog double positive.

Supplement Figure 1: Overall survival of patients with oral squamous cell carcinoma according to Stage (a), Lymph Node Metastasis (c) and Histological grade (e); Disease-free survival of patients with oral squamous cell carcinoma according to Stage (b), Lymph Node Metastasis (d) and Histological grade (f) calculated by Kaplan-Meier method. Negative – includes categories 0 and 1⁺; Positive – includes categories 2⁺ and 3⁺; Mod. + Poor – moderate and poorly differentiated tumors; Well – well differentiated tumours.

Supplement Figure 2: The co-localization between β -catenin and Oct-4/Nanog was analyzed in H314 cells. A. Expression of Oct-4 (a), β -catenin (b), β -catenin + Oct-4 (c), DAPI (d), β -catenin + Oct-4 + DAPI (e) and β -catenin + DAPI (f) in H314 cell. B. Expression of Nanog (a), β -catenin (b), β -catenin + Nanog (c), DAPI (d), β -catenin + Nanog + DAPI (e) and β -catenin + DAPI (f) in H314 cell. Yellow indicates Co-localization of β -catenin (red) with Oct-4 / Nanog (green). C. Intracellular localization of β -catenin (a) and β -catenin + DAPI (b) in Oral carcinoma tissue.

Table: 1
Immunodetection of β -catenin, Oct-4 and Nanog in oral squamous cell carcinoma.

Protein	No. of Cases	0	1+	2+	3+
β -catenin	60	10	9	11	30
Oct-4	60	20	12	10	18
Nanog	60	17	14	10	19

0 (negative), no staining or <5% positive cells; 1⁺ (mild) , 5-15% positive cells; 2⁺ (moderate), 16-25% positive cells and 3⁺ (intense), >25% positive cells.

Table 2. Correlation of β -Catenin, Oct and Nanog expression with clinicopathological factors

	No. of patients	β -Catenin			Oct-4			Nanog		
		Negative(19)	Positive(41)	χ^2 / p-value	Negative(32)	Positive(28)	χ^2 / p-value	Negative(31)	Positive(29)	χ^2 / p-value
<u>Age</u>										
<60	32	11	21	0.232 / p<0.63	17	15	0.001 / p<0.97	16	16	0.076 / p<0.78
\geq 60	28	8	20		15	13		15	13	
<u>Gender</u>										
Male	34	12	22	0.477 / p<0.49	18	16	0.005 / p<0.94	17	17	0.087 / p<0.76
Female	26	7	19		14	12		14	12	
<u>Stage</u>										
I-II	27	13	14	6.163 / p<0.01*	19	8	5.725 / p<0.01*	19	8	6.877 / p<0.009**
III-IV	33	6	27		13	20		12	21	
<u>Lymph node Metastasis</u>										
Negative	28	14	14	8.155 / p<0.004**	20	8	6.907 / p<0.009**	20	8	8.210 / p<0.004**
Positive	32	5	27		12	20		11	21	
<u>Histological Grade</u>										
Well Differentiated	16	9	7	6.181 / p<0.04*	15	1	15.265 / p<0.001**	15	1	18.204 / p<0.001**
Moderately Differentiated	20	5	15		11	9		10	10	
Poorly Differentiated	24	5	19		6	18		6	18	
<u>Location of the tumours</u>										
Tongue	20	6	14	0.275 / p<0.99	12	8	1.470 / p<0.83	12	8	3.525 / p<0.47
Buccal	9	3	6		4	5		4	5	
Palate	5	2	3		3	2		2	3	
Gingiva	14	4	10		8	6		9	5	
Floor of mouth	12	4	8		5	7		4	8	

Negative (includes category 0 and 1⁺) and Positive (includes category 2⁺ and 3⁺).

* Statistically significant, ** Statistically highly significant.

Table 3: Correlation between the expression pattern of β -Catenin, Oct-4 and Nanog in oral cancer.

Expression of β -Catenin		Expression of Oct-4				Expression of Nanog				
		(No. of patients = 60)	0 (20)	1 ⁺ (12)	2 ⁺ (10)	3 ⁺ (18)	0 (17)	1 ⁺ (14)	2 ⁺ (10)	3 ⁺ (19)
0	10		9	1	0	0	7	2	1	0
1 ⁺	9		6	2	1	0	5	2	2	0
2 ⁺	11		5	3	3	0	4	4	3	0
3 ⁺	30		0	6	6	18 ^a	1	6	4	19 ^b

0 (negative), no staining or <5% positive cells; 1⁺ (mild), 5-15% positive cells; 2⁺ (moderate), 16-25% positive cells and 3⁺ (intense), >25% positive cells.

a = Significant correlation was found between expression of β -Catenin and Oct-4 ($p < 0.001^{**}$, $r = 0.80$).

b = Significant correlation was found between expression of β -Catenin and Nanog ($p < 0.001^{**}$, $r = 0.71$).

^{**} = Statistically highly significant.

Table 4: Correlation of β -Catenin localization with the expression patterns of Oct-4 and Nanog in oral squamous cell carcinoma.

Localization of β -Catenin		Expression of Oct-4			Expression of Nanog		
		0+1 (32)	2(10)	3(18)	0+1 (31)	2(10)	3(19)
(No. of patients = 60)							
0	19	18	1	0	16	3	0
M	11	8	3	0	9	2	0
I	30	6	6	18 ^a	6	5	19 ^b

0+1(negative/mild; including categories 0 and 1⁺), 2 (moderate; including category 2⁺) and 3 (intense; including category 3⁺).

0, negative; M, membranous and I, intra-cellular.

a = Significant correlation was found between localization of β -Catenin and expression of Oct-4 ($p < 0.001^{**}$, $r = 0.71$).

b = Significant correlation was found between localization of β -Catenin and expression of Nanog ($p < 0.001^{**}$, $r = 0.67$).

**** Statistically highly significant.**

Table 5. Kaplan-Meier and log-rank analysis for Clinicopathological variables, β -catenin, Oct-4 and Nanog in relation to Overall and Disease-free survival of 60 patients with Oral Squamous Cell Carcinoma.

Variables	Overall Survival	Disease-free Survival
	p value	p value
1. Stage (I, II /III, IV)	0.0003**	0.002**
2. Grade (Well/Moderate,Poor)	0.018*	0.0009**
3. LN Metastasis (+/-)	<0.001**	0.041*
4. β -catenin (+/-)	0.003**	0.039*
5. Oct-4 (+/-)	<0.001**	<0.001**
6. Nanog (+/-)	<0.001**	0.001**
7. β -catenin+Oct-4 (B ⁺ /O ⁺ vs others)	<0.001**	<0.001**
8. β -catenin+Nanog (B ⁺ /N ⁺ vs others)	<0.001**	<0.001**
9. β -catenin localization (Intracellular vs others)	<0.001**	<0.001**

- = Includes category 0 and 1⁺, + = Includes category 2⁺ and 3⁺, LN = Lymph node,

B⁺/O⁺ = β -catenin and Oct-4 double positive cases, B⁺/N⁺ = β -catenin and Nanog double positive cases.

* Statistically significant, ** Statistically highly significant.

Table 6. Univariate Cox proportional hazards regression analysis (Cox Method) for variables in relation to Overall and Disease-Free Survival of 60 patients with Oral Squamous Cell Carcinoma.

Variables	Overall Survival			Disease-free Survival		
	p value	Hazards Ratio	95% CI	p value	Hazards Ratio	95% CI
1. Stage (I, II /III, IV)	0.003**	8.966	2.069-38.857	0.001**	4.712	1.930-11.506
2. Grade (Well/Moderate, Poor)	0.047*	0.130	0.017-0.976	0.005**	0.129	0.030-0.543
3. LN Metastasis (+/-)	0.003**	2.074	0.276-15.569	0.048*	2.085	1.007-4.317
4. β -catenin (+/-)	0.021*	1.076	0.143-8.078	0.048*	2.349	1.008-5.477
5. Oct-4 (+/-)	<0.001**	15.015	3.440-65.530	<0.001**	7.764	3.256-18.516
6. Nanog (+/-)	0.001**	7.716	2.243-26.544	0.002**	3.222	1.514-6.860

- = Includes category 0 and 1⁺, + = Includes category 2⁺ and 3⁺, LN = Lymph node.

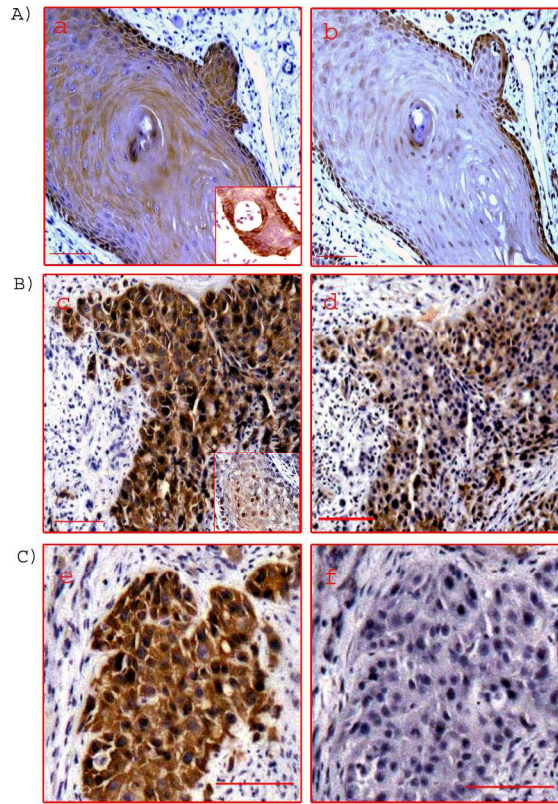
* Statistically significant, ** Statistically highly significant.

Table 7. Multivariate Cox proportional hazards regression analysis (Cox Method) for variables in relation to Overall and Disease-Free Survival of 60 patients with Oral Squamous Cell Carcinoma.

Variables	Overall Survival			Disease-free Survival		
	p value	Hazards Ratio	95% CI	p value	Hazards Ratio	95% CI
1. Stage (I, II /III, IV)	0.015*	7.781	1.491-40.617	0.024*	3.254	1.165-9.091
2. β -catenin	0.018*	1.403	0.561-12.621	0.008**	4.268	1.458-12.490
3. Oct-4	0.022*	7.236	1.334-39.243	0.001**	9.288	2.982-28.930
4. Nanog	0.025*	5.431	1.235-23.892	0.005**	4.591	1.578-13.356

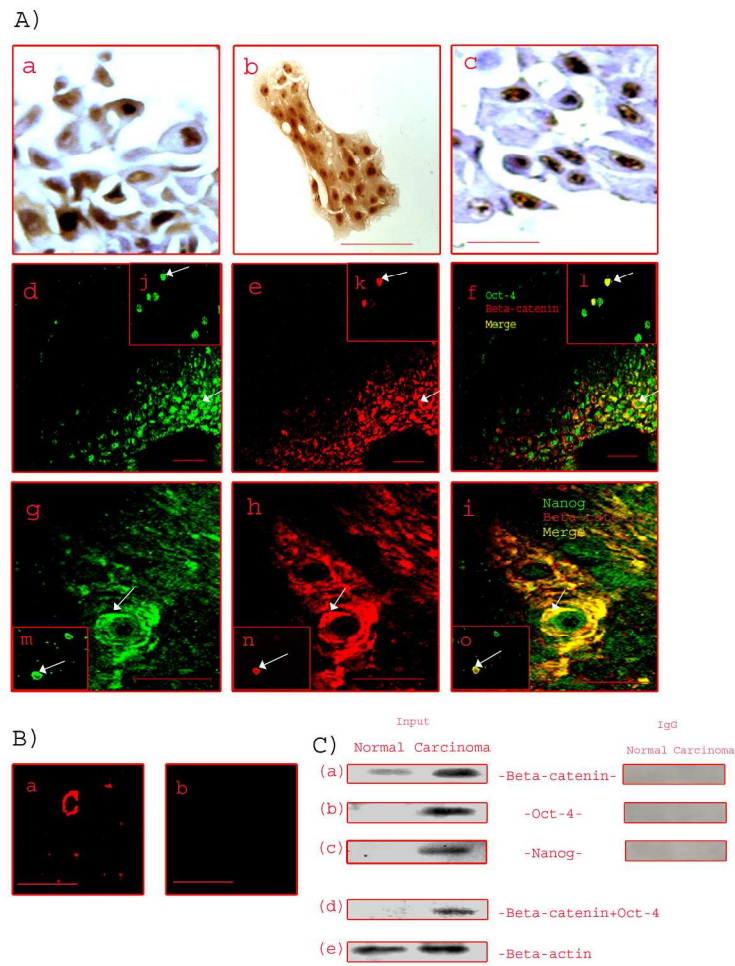
* Statistically significant, ** Statistically highly significant

Figure 1



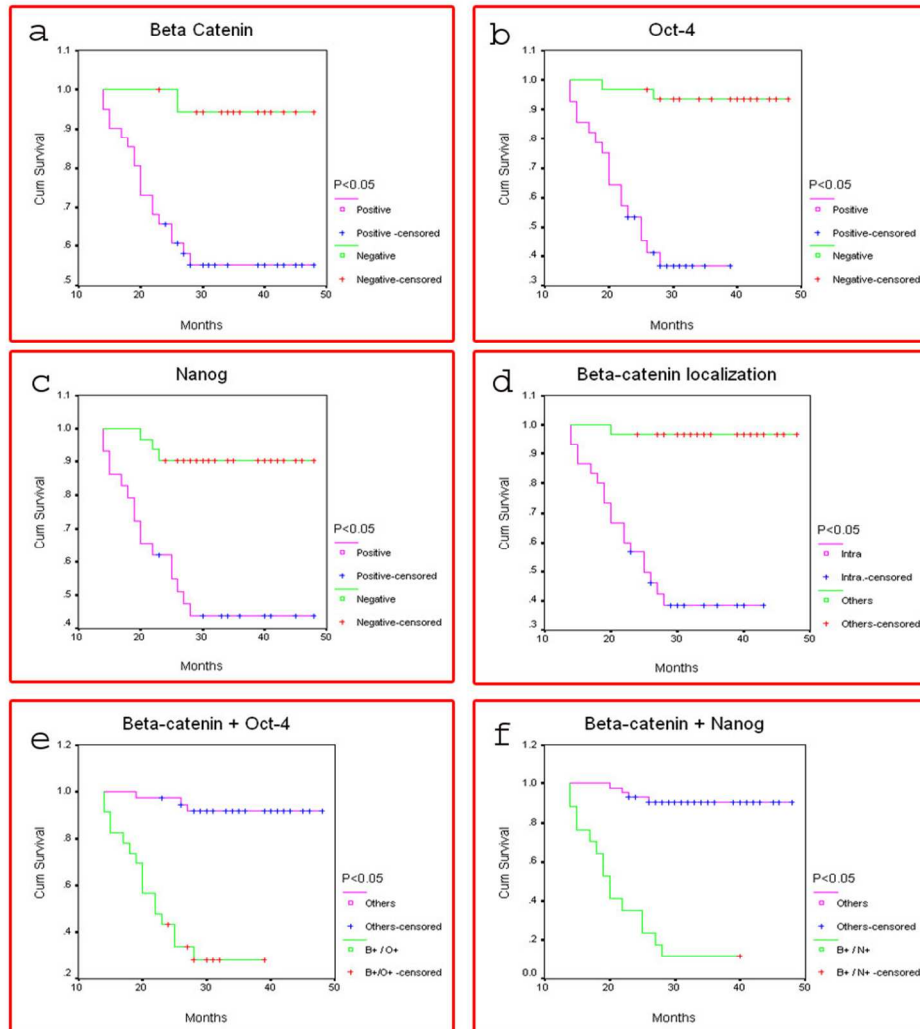
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Figure 2



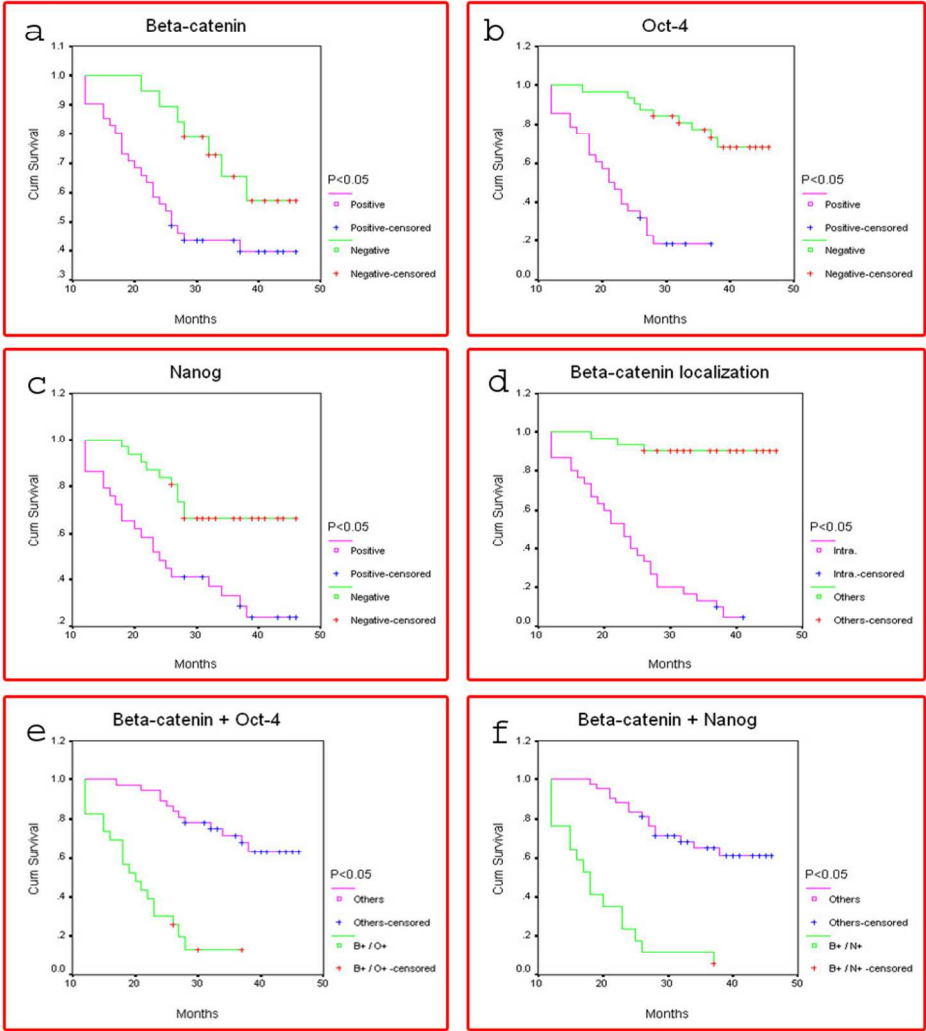
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Figure 3:



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Figure 4



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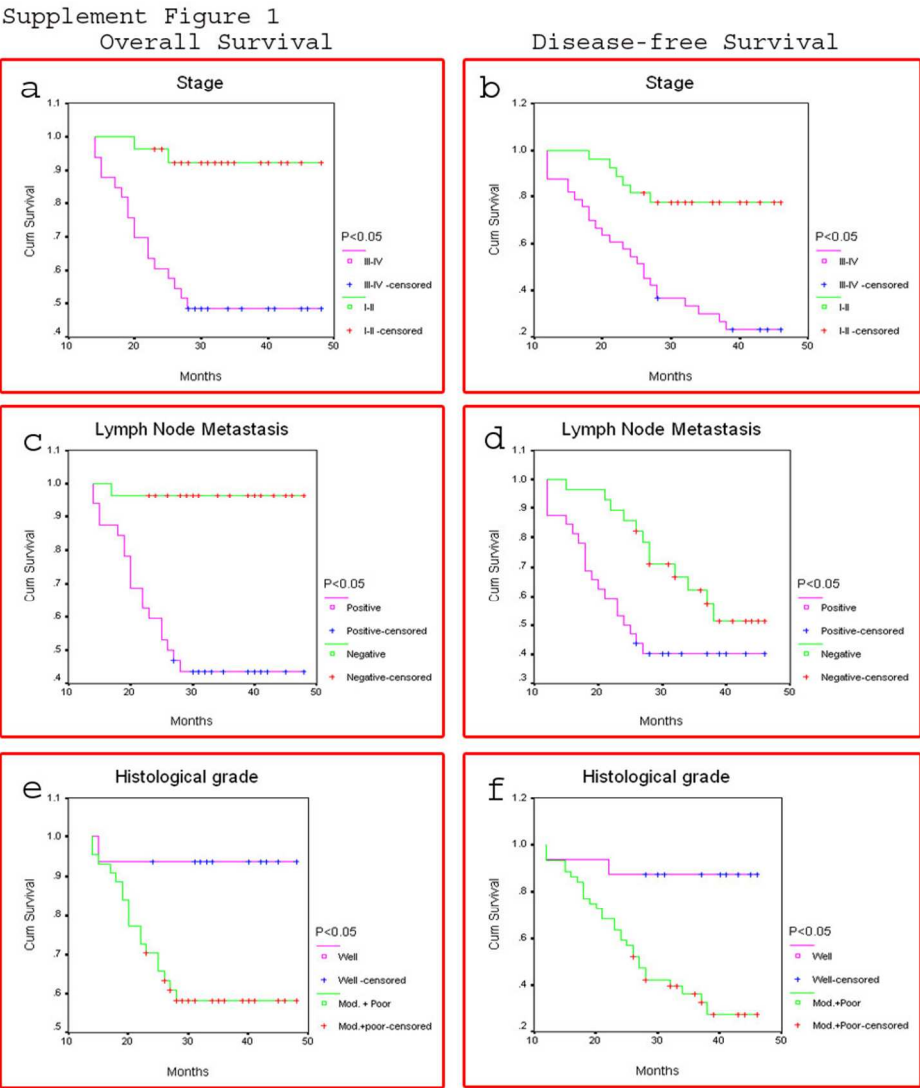
Supplement table 1

Comparison of β -catenin – Oct-4 interaction between Normal and Carcinoma.

Group	n	β -catenin – Oct-4	
		Negative	Positive
1. Normal	6	6	0
2. Carcinoma	10	2	8 ^a

a = Significant difference was found between Normal and Carcinoma cases ($p < 0.002^{**}$).

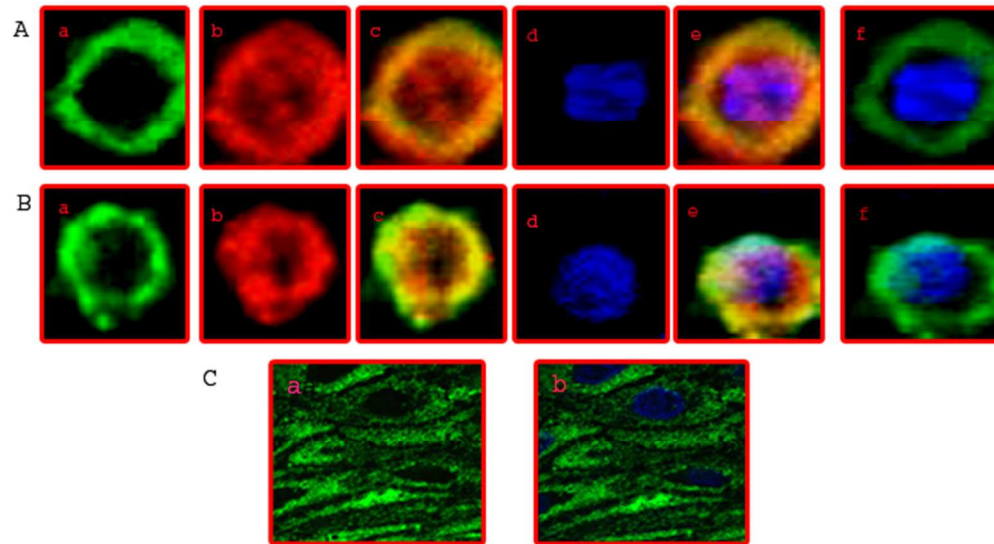
** = Statistically highly significant.



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Supplement Figure 2



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